[19] State Intellectual Property Office of the People's Republic of China

[51] Int. Cl[7].
**A61K 31 / 704**
A61P 31 / 12 A61P 9 / 00

**[12] Invention Patent Specifications**

**[21] ZL Patent No.: 00119451.8**

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| **[43] Publication Date:** April 14, 2001 | **[11] Publication No.:** CN 1283462A |
| **[22] Application Date:** July 14, 2001<br>[21] **Application No.: 00119451.8** | **[74] Patent Agency:** Shanghai Medical University Patent Office<br>**Agent:** Wu Guiqin |
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| | Number of pages of claims: 1<br>Number of pages of specifications: 7<br>Number of pages of drawings: 1 |


**[54]** Name of the invention: the Utility of Astragaloside IV in Preparing for Composite Drugs

**[57] Abstract**

The present invention provides Astragaloside IV, a substance extracted from Traditional Chinese Medicine ingredient called astragalus, as well as its preparation method, including the preparation of astragalus concentrate, refined astragalus solution and the separation process of Astragaloside IV. At the same time, the present invention provides the tablets, injection agent, slow release agent, capsules and the compound agent made from the raw material of Astragaloside IV. The present invention also touches upon the application of this extract in treating viral myocarditis.

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## Claims

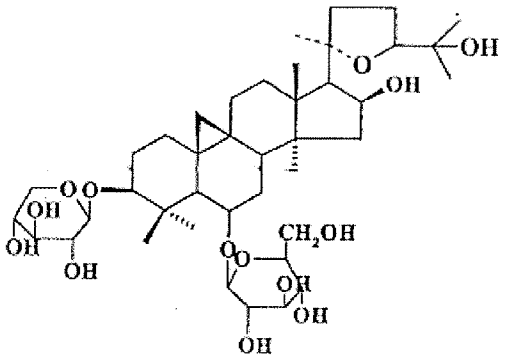
1. The application of Astragaloside IV, an extract from astragalus, in the preparation of the composite drug. Astragalus is an ingredient of Traditional Chinese Medicine.
2. According to Claim 1, this involves the application of Astragaloside IV of Traditional Chinese Medicine in the preparation of a composite drug used to treat viral myocarditis.
3. According to Claim 1 and Claim 2, Astragaloside IV can be used as a raw drug material to prepare for a variety of dosage forms, such as tablets, injection agent, slow release pills, capsules, and compound drug agent.

The application of Astragaloside IV in the preparation of composite drugs

The present invention involves the pharmaceutical utility of Astragaloside IV, an extract from Traditional Chinese Medicine ingredient called astragalus. Specifically the present invention touches upon the application of Astragaloside IV in the preparation of composite drugs used to treat viral myocarditis.

Viral myocarditis is a common heart disease in the clinical medicine. This disease is a threat to human lives. An early detection and treatment can save lives. As a common treatment drug for viral myocarditis, astragalus of Traditional Chinese Medicine used as a complete drug in clinical treatment has scored a good curative result. At present, however, the quality control for astragalus injection solution is pretty difficult due to a variety of impacting factors. Deposits are easily formed in the astragalus injection solution, leading to the loss of curative efficacy. It has been a strong desire of clinical doctors and pharmacists to seek an astragalus drug that is stable in quality, appropriate in price and efficacious in curative effect.

Astragaloside IV is an extract separated from astragalus, a common ingredient of Traditional Chinese Medicine. Astragaloside IV is a monomeric compound and its chemical compound structure is shown as follows:




The molecular formula $C_{41}H_{68}O_{14}$

The crystal form colorless needle like crystal

The melting point 309 -- 310°C

The solubility It has a slight solubility in methanol, but has almost no solubility in acetic ether, acetone and water. It has solubility in ethanol when ethanol is heated and it is separated out when ethanol gets cold.

Its infrared spectrum (KBr) $cm^{-1}$ is 3510, 3388, 2950, 2870, 1650, 1458, 1380, 1367, 1070, 1047, 1020, 895.

The purpose of the present invention is to provide a monomeric compound of Astragaloside IV as a raw material in the preparation of pharmaceutical products.

Another purpose of the present invention is to provide new utilities of Astragaloside IV, that is, the new utilities in the preparation of drug forms used to treat viral myocarditis. It touches upon the application of Astragaloside IV in the preparation of drugs used to prevent and treat viral myocarditis caused by coxsackie virus.

The present invention prepares for Astragaloside IV by means of the following method and steps:

(1) Prepare for astragalus concentrate
Get ready astragalus medical plants, get rid of impurities, slice them into pieces, add in water of ten fold volume, let astragalus slices soak in water for 30 minutes, and then stew them for one hour and a half, filter them, and preserve the first batch of stewed liquid. Throw in water of eight fold volume to the astragalus dregs and stew them for one hour, filter them, and preserve the second batch of stewed liquid. Again throw in water of eight fold volume to the astragalus dregs and stew them for one hour, filter them, and preserve the third batch of stewed liquid. Then put together all batches of stewed liquid, condense the stewed liquid under regular pressure to a concentrate solution of approximately 2 g raw astragalus drug per ml.

(2) Prepare for refined astragalus solution
Throw in ethanol to the aforesaid astragalus concentrate solution to let the ethanol concentration reach 70%, stir the solution compound and place it at 2 -- 10°C condition for 48 to 72 hours. Filter the astragalus solution, get rid of deposits, retrieve ethanol from the filtered solution, and condense the solution to a raw drug concentrate of approximately 2 g of astragalus per ml. Then throw in ethanol to let ethanol concentration reach 80%, stir the solution compound and place it at 2 - - 10°C condition for 48 to 72 hours. Filter the solution and get rid of deposits, retrieve ethanol from the filtered solution, and condense the solution to a thick extract. Add in distilled water of 6 to 7 fold of volume to the thick concrete, stir and mix them evenly, and place the mixture at 2 - - 10°C condition for 12 hours. Filter out any deposits, add in an appropriate amount of active carbon to the filtered liquid and boil it for 30 minutes. Let the liquid cool down to the room temperature, filter out active carbon and deposits. Adjust the PH value of the liquid to 7.5 by means of NAOH, thus obtaining the refined astragalus solution.

(3) The separation of Astragaloside IV
Extract three times from the aforesaid refined astragalus solution by means of an equal volume of n - butyl alcohol. Put together the liquids from the three extractions to get n - butyl alcohol extract liquid. Retrieve the solvent medium to obtain astragalus n - butyl alcohol concrete.

Dissolve the aforesaid astragalus n - butyl alcohol concrete into a small volume of methanol, throw in silica gel for laminar analysis. Stir and mix them evenly, shake the methanol dry above the water bath, thus obtaining the liquid like compound between astragalus n - butyl alcohol concrete and silica gel.

Pour the liquid like compound to the top of a silica gel column, and make laminar analysis of the compound by means of silica gel thin film. Wash it out by means of chlorofom / methanol in gradient, make laminar analysis by thin film, conbime the parts containing Astragaloside IV, retrieve the

solvent medium and separate the white solid substance. Re - crystallize the white solid substance by means of methanol, let it sit for a while to separate colorless needle like Astragaloside IV crystal.

Astragaloside IV of the present invention can be used as a drug raw material to be prepared for tablets, injection agent, slow release pills, capsules as well as compound drug forms.

Through animal test, Astragaloside IV of the present invention has been proven to be efficacious in curing viral myocarditis on small rats caused by coxsackie $B_3$ virus, to be able to reduce myocarditis area, to alleviate necrosis extent caused by myocarditis, and to improve the heart function. Astragaloside IV is proven to protect the heart muscle cells infected by $CVB_3$, and is able to resist coxsackie virus.

The animal test method and test results of the present invention are described as follows:
I. The experimental research into applying Astragaloside IV in treating viral myocarditis on small rats caused by coxsackie $B_3$ virus and the impact of Astragaloside IV on the granular enzyme B expressions

(1) The drug source and drug administration channels

Astragaloside IV, extracted and purified by the Pharmaceutical Section of Zhongshan Hospital, is a colorless needle like crystal substance. It does not dissolve into water. Add Astragaloside IV into the water solution with 5% CMC, with a concentration of 1 mg / ml, 2mg / ml, 4mg / ml respectively. Astragalus injection solution (provided by Shanghai Fuda Pharmaceutical Co. Ltd., each injection tube 4g / 2ml) is given by intragastric administration to small rats on the day they were inoculated with virus in the belly. Each administration contains 0.1ml, once a day. The normal comparison and virus comparison group small rats are given by intragastric administration water solution with 5% CMC.

(2) Experimental animals and their groups

Take 120 male 4 - week old BALB / c small rats with a clean grade, and put them into normal comparison group, normal + astragalus group, normal + Astragaloside IV group; Virus comparison group, virus + astragalus (astragalus injection solution 0.2g) group, virus + Astragaloside IV 0.1 mg, 0.2 mg and 0.4 mg groups.

(3) Observe the function of the drug toxicity

Compare normal + astragalus (astragalus injection solution 0.2g) group small rats with normal + Astragaloside IV (0.4mg) group small rats and normal group small rats. Observe their general conditions and find out any possible deaths. Kill the experimental small rats a week later, take out their hearts, stabilize the small rat hearts with 4% paraformic aldehyde, wrap them up with mineral wax and slice them into pieces with each slice thickness of 4 μm, run a HE dyeing examination to observe any pathological changes in the heart muscle.

(4) Set up small rat viral myocarditis model caused by coxsackie $B_3$ virus

1. Virus: Coxsackie $B_3$ virus, Nancy strain, provided by this lab. After Vero cells have multiplied the strain numbers, the titer of the virus is $10^{10}TCID_{50}$.

2. The experimental animals: Male 4 - week old BALB / c small rats with a clean grade, provided by the Experimental Animal Center of Shanghai Medical University.

3. Animal model preparation: Inoculate bellies of the experimental group small rats with 0.1ml viral Eagle liquid which contains $10^5$ $TCID_{50}$ coxsackie $B_3$ virus; inoculate bellies of the comparison group small rats with 0.1ml Eagle liquid which contains no virus. Observe dietary situations, fur color changes and activities of small rats, check to see if there are any deaths. Seven days after the inoculation, kill all the small rats, take out their hearts, make a regular check on pathological changes, check on hybridization in situ as well as check on immuno histochemical conditions.

(5) Observe the drug efficacy

Compare the treatment group small rats with non - treatment group small rats to find out death rate, disease rate, the pathological changes, make a quantifiable analysis of the pathological results, and compare the area percentages of pathological changes caused by myocarditis of all groups.

(6) Inspect the granular enzyme B expressions in the heart muscle of small rat by means of immuno histochemical method

The results:

(I) The drug toxicity function shows a similarity between the normal + Astragalus group and the normal + Astragaloside IV 0.4 mg group small rats and the normal comparison group small rats. The general conditions of those small rats are fine, with no deaths. No pathological changes in the heart muscle are found.

(II) The pathological changes of myocarditis among small rats are found. Compared with the normal group small rats, the conditions of the small rats in the group infected with virus are much worse in that they eat less and move less, some small rats have died. The heart pathological checkup has found myocarditis occurrences of varying degrees.

Table 1 shows the comparison results of the pathological changes in the heart muscle of small rat

| Group | Sequence No of small rats | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| Virus comparison | 1 | 0.5 | 40 | 1 | 15 | 70 | 30 | 3 | 60 | |
| Virus + astragalus | 3 | 5 | 0 | 30 | 10 | 1 | 50 | 8 | | |
| Virus + Astragaloside IV 0.1 mg | 25 | 1 | 0 | 1 | 55 | 3 | 7 | | | |
| Virus + Astragaloside IV 0.2 mg | 0 | 10 | 6 | 10 | 3 | 20 | 1 | 1.5 | 1 | |
| Virus + Astragaloside IV 0.4 mg | 4 | 0 | 15 | 1 | 1 | 4 | 8 | 0 | 0 | 6 |

These results show there is a large constituent ratio of serious pathological change in the heart muscle of the non - treatment group small rats, while there is a small constituent ratio of light pathological change in the animal heart muscle. The pathological change among small rats in the treatment groups of Astragaloside IV 0.2 mg group and the Astragaloside IV 0.4 group is lighter than those in the non - treatment group, with $p < 0.05$.

Table 2: The frequency and number of the pathological change area in the small rat heart muscle

| | | Pathological change area | (%) | 75 - 100 | Total |
|---|---|---|---|---|---|
| Group | 0 - 25 | 25 - 50 | 50 - 75 | | |
| Virus comparison | 5 | 2 | 2 | 0 | 9 |
| Virus + astragalus | 6 | 1 | 1 | 0 | 8 |
| Virus + Astragaloside IV 0.1 mg | 5 | 1 | 1 | 0 | 7 |
| Virus + Astragaloside IV 0.2 mg | 9 | 0 | 0 | 0 | 9 |
| Virus + Astragaloside IV 0.4 mg | 10 | 0 | 0 | 0 | 10 |

Table 2 shows a downward trend in the pathological change area among the small rats in the treatment group, especially in the Astragaloside IV 0.2 treatment group and the Astragaloside IV 0.4 treatment group, in which the pathological change area in the small rat heart muscle is invariably < 25%. We can draw a demarcation line on 25% of the pathological change area in the heart muscle, and in this way we will be able to calculate both serious and light constituent ratios of the pathological changes for all groups. The serious and light constituent ratios of the pathological change in the heart muscle in the Astragaloside IV 0.2 treatment group is 9 / 10. Compared with the virus comparison group with a constituent ratio of 5 / 4, there is an obvious difference, $p = 0.04$. The constituent ratio of the pathological change in the heart muscle in the Astragaloside IV 0.4 treatment group is 10 / 0. Compared with the virus comparison group with a constituent ratio of 5 / 4, there is an obvious difference, $p = 0.03$. Compared with the virus comparison group, the rest other groups do not exhibit an obvious difference in the constituent ratio in the pathological change in the heart muscle.

(III) The examination by means of immuno histochemical method shows a positive ratio between the granular enzyme B expressions and the pathological change area. The pathological change area is smaller for the treatment groups (the Astragaloside IV 0.2 group and the Astragaloside IV 0.4 group), and the granular enzyme B expressions are also low. No granular enzyme B expressions have been found in the heart muscle of small rats free from pathological myocarditis.

II. The effect of Astragaloside IV on the heart function of small rats with viral myocarditis

(1) Set up the model of viral myocarditis for small rats. After the small rats are infected with virus, perform an electrocardiogram exam on the VMC small rats. The exam shows a shrinking FS score in the left ventricle, the peak systolic velocity of the blood stream in the main artery is Vp, and the accumulated score of blood flow velocity in the main artery is Vi. After an ultrasound check is performed on the surviving small rats, they are killed, and a pathological checkup is performed on their hearts so as to observe the dynamic changes and pathological changes in all heart function indicators.

(2) The VMC model of small rats is divided into the Astragaloside IV group, the astragalus group, the blank comparison group and the normal comparison group. Seven days after the virus is inoculated into their bodies, an electrocardiogram examination is performed on the surviving small rats and a score is given to the heart pathology. At the same time, ELISA measurement method is used to determine the content of troponin in the blood serum. All small rats are grouped based on their accumulated pathological scores, a score $< 1.5$ indicates a light pathological condition, a score $\geq 1.5$ indicates a serious pathological condition. An examination of hybridization in situ in performed on the mineral wax slices of the mid section of the heart to detect virus RNA, and to calculate the percentage of positive signals under the light microscope compared with the total area.

The results:

(1) Small rats in VMC group have died. Compared with the normal comparison group their heart function indicative figures are low.

(2) FS, Vp and Vi indicative figures in VMC group are all very low compared with the comparison - normal group. This indicates the weakening heart function in the virus - infected group. The heart function exhibits an obvious decline in the virus - infected group without being administered the drug ($p < 0.05$).

(3) The pathological scoring: The comparison - VMC group has a large necrosis area, while the Astragaloside IV - VMC group has a smaller necrosis area, mainly showing symptoms of inflammation infiltration. The pathological change in the astragalus - VMC group is similar to that of the Astragaloside IV - VMC group. The pathological change in the non - treatment group is worse than that of the treatment group ($p < 0.05$). No pathological changes are detected in the comparison - normal group and the Astragaloside IV - normal group, with an accumulated score of 0. Compared with the previous three groups, these two groups exhibit a distinct difference ($p < 0.05$).

These results have ascertained that Astragaloside IV is able to alleviate the extent of necrosis of MVC group small rats with myocarditis conditions, and improve the heart function.

III. Astragaloside IV can protect *in vitro* viral myocarditis cells

1. Selection of virus: Coxsachie $B_3$ virus is selected as a source of infection. Reed method is used to measure 50% infection rate ($TCID_{50}$) in the heart muscle cells of suckling rats.

2. Prepare and cultivate heart muscle cells: Take Sprague - Dawley big rats (provided by the experimental animal department of Shanghai Medical University), and digest cells in a series in the heart ventricle by means of 0.1% trypsin. The suspension cell solution thus obtained are purified and cultivated. Eagle's MEM of the calf blood serum at 20% concentration is used as a life sustaining solution.

3. Observe the impact of virus on the cultivation of the heart pulsation cells and any changes in morphology: Inocluate an appropriate amount of 100 TCID50 Coxsachie $B_3$ virus to the heart muscle cells that show a regular pulsation, let cells and virus absorb each other for one hour at 37°C condition, and observe any possible changes. The result shows that two days after the virus inoculation, the percentage of heart muscle pulsation declines significantly. CPE changes quickly from + to 4+, the cells change to round shape, heap together, showing refraction and shrinking. CPE expression method is : + indicating < 25% CPE, 1+ indicating 25% CPE, 2+ indicating 50% CPE, 3+ indicating 75&CPE, 4+ indicating almost 100% CPE.

4. Drug treatment: Place the prepared heart muscle cells into three groups: the normal comparison group, the virus comparison group, the virus + Astragaloside IV. The aforesaid life sustaining solution is only added to the normal group and virus - infected group, while the life sustaining solution of 0.1 mg / ml concentration is added to the astragalus treatment group. Put all three groups at 37 °C condition for hatching, and observe under the inversion difference microscope and record any changes.

The observed results show that Astragaloside IV is not only able to improve the percentage of pulsation cells infected with CVB3 virus, but also protect the cell form.

The description of the attached drawings:

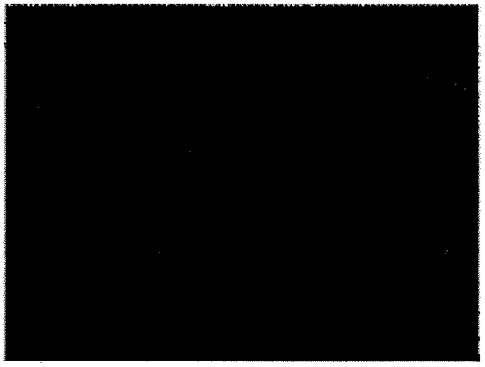
Graph 1 The protection provided by Astragaloside IV for *in vitro* viral myocarditis cells

Drawing description

Graph 1

Virus + Astragaloside IV treatment

Virus + astragalus injection solution comparison



Virus comparison

Normal astragalus comparison

Normal cells

[19]中华人民共和国国家知识产权局

[51]Int. Cl[7]
A61K 31/704
A61P 31/12 A61P 9/00

# [12]发明专利申请公开说明书

[21]申请号 00119451.8

[43]公开日 2001年2月14日

[11]公开号 CN 1283462A

[22]申请日 2000.7.14 [21]申请号 00119451.8
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权利要求书1页 说明书7页 附图页数1页

[54]发明名称 黄芪甲苷在制备药物组合物中的应用

[57]摘要

本发明提供一种中药黄芪提取物黄芪甲苷,及其制备方法。包括制备黄芪浓缩 液、黄芪精制液、分离黄芪甲苷;同时提供此提取物可作为药物原料制成片剂、注射剂、缓释剂、胶囊和复方制剂;还涉及提取物在治疗病毒性心肌炎中的用 途。

ISSN1008-4274

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# 权 利 要 求 书

1. 中药黄芪提取物黄芪甲苷，在制备药物组合物中的应用。

2. 按权利要求 1 所述的中药黄芪甲苷，在制备治疗病毒性心肌炎药物组合物中的应用。

3. 按权利要求 1 和 2 所述的黄芪甲苷可作为药物原料，制成片剂、注射剂、缓释剂、胶囊和复方制剂等剂型。

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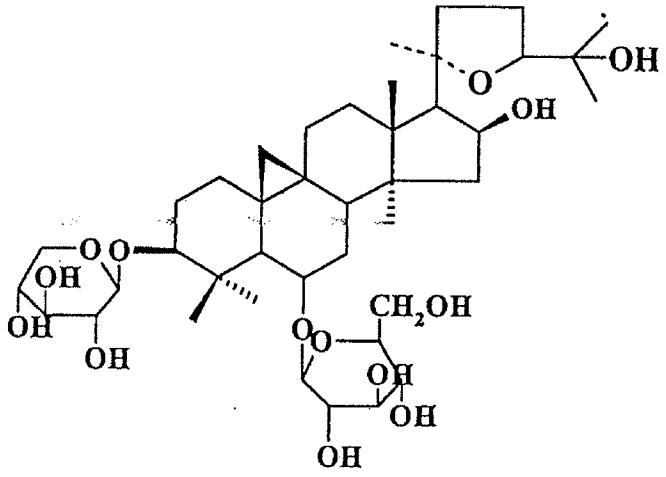
# 说　明　书

## 黄芪甲苷在制备药物组合物中的应用

本发明涉及中药黄芪提取物黄芪甲苷的药物用途，具体涉及黄芪甲苷在制备治疗病毒性心肌炎药物中的应用。

病毒性心肌炎是临床常见心脏疾病，常危及病人生命安全，需及早发现、早日治疗。中药黄芪作为常用治疗药，其全药应用于临床治疗，已取得较好效果，但目前治疗用黄芪注射液，因外界各种因素影响，其质量不易控制，易产生沉淀，而影响治疗效果，寻找一种质量稳定、价格适宜、治疗效果好的中药治疗剂，一直是临床医生和药师的愿望。

黄芪甲苷（astragaloside Ⅳ）是自中药黄芪中提取、分离的单体化合物，其化合结构式如下:




分子式　$C_{41}H_{68}O_{14}$

晶型　无色针状结晶

熔点　309-310℃

溶解度　甲醇稍溶，乙酸乙酯、丙酮及水几乎不溶，乙醇加热溶解，冷后析出。

红外光谱（KBr）$cm^{-1}$ 3510，3388，2950，2870，1650，1458，1380，1367，1070，1047，1020，895

本发明的目的是提供黄芪甲苷单体化合物作为药物制剂的原料。

本发明的另一目的是提供黄芪甲苷的新用途，即在制备防治病毒性疾病药物制剂的用途。涉及黄芪甲苷作为制备预防和治疗柯萨奇病毒性心肌炎药物组合物的应用。

本发明通过下述方法和步骤制备黄芪甲苷:

（1）制备黄芪浓缩液

取黄芪药材，去除杂质后，切片，加 10 倍量的水，浸泡 30 分钟后煎煮 1.5 小时，过滤，保留第一次煎煮液，药渣再加 8 倍量的水煎煮 1 小时，过滤，保留第二次煎煮液，药渣再加 8 倍量的水煎煮 1 小时，过滤，保留第三次煎煮液，然后合并三次煎煮液，常压浓缩至每毫升相当于含 2 克黄芪生药的浓缩液。

（2）制备黄芪精制液

将上述黄芪浓缩液加入乙醇至含醇量为 70%，搅拌后于 2—10℃条件下放置 48—72 小时，过滤，去除沉淀，滤液回收乙醇并浓缩至每毫升相当于含 2 克黄芪生药的浓缩液，再加入乙醇至含醇量为 80%，搅拌后于 2—10℃条件下放置 48—72 小时，过滤，去除沉淀，滤液回收乙醇并浓缩至稠浸膏，加稠浸膏量的 6—7 倍的蒸溜水，搅拌均匀，于 2—10℃条件下放置 12 小时，滤除沉淀，滤液加适量活性碳煮沸 30 分钟，冷至室温，于 2—10℃条件下放置 12 小时，滤除活性碳和沉淀，滤液用 NAOH 调 PH 为 7.5，制得黄芪精制液。

（3）分离黄芪甲苷

将上述黄芪精制液，用等量正丁醇提取 3 次，合并 3 次提取液得正丁醇提取液，回收溶媒，制得黄芪正丁醇浸膏。

将上述正丁醇浸膏，用少量甲醇溶解，加入层析用硅胶，搅匀，水浴上挥干甲醇，制得呈流动状的黄芪正丁醇浸膏与硅胶的混合物。

将其加入到硅胶柱顶部，以硅胶簿层层析检视，用氯仿/甲醇梯度洗脱，簿层层析检视，合并有黄芪甲苷的部分，回收溶媒，析出

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白色固体物。将白色固体物用甲醇重结晶，放置，析出无色针状结晶黄芪甲苷。

本发明黄芪甲苷可作为药物原料制成片剂、注射剂、缓释剂、胶囊以及复方制剂。

本发明黄芪甲苷经动物实验，结果证实对小鼠柯萨奇 $B_3$ 病毒性心肌炎有治疗作用，能减少心肌炎面积，减轻 VMC 小鼠心肌炎坏死程度改善心功能。对 $CVB_3$ 感染的心肌细胞具有良好的保护作用。有抗柯萨奇病毒作用。

本发明的动物实验方法与结果如下:

一、黄芪甲苷治疗小鼠柯萨奇 $B_3$ 病毒性心肌炎的实验研究及其对颗粒酶 B 表达的影响

（一）药物来源及给药途径

黄芪甲苷 ，由中山医院药剂科分离提纯，为无色针状结晶 ，不溶于水，将黄芪甲苷加入到 5%羧甲基纤维素钠 （CMC）水溶液中，浓度分别为 1 mg/ml 、2mg/ml 、4mg/ml ，黄芪注射液（上海福达制药有限公司提供，每支 4g/2ml ），自小鼠腹腔注射病毒日起 ，灌胃给药，每日 1 次，每次 0.1ml，正常及病毒对照组小鼠灌胃给 0.1ml 的 5%羧甲基纤维素钠水溶液。

（二）实验动物及分组

清洁级 4 周龄 BALB/c 雄性小鼠 120 只，分为正常对照组、正常+黄芪组，正常+黄芪甲苷组；病毒对照组，病毒+黄芪（黄芪注射液 0.2g）组，病毒+黄芪甲苷 0.1mg 、0.2mg 、0.4mg 组。

（三）观察药物毒性作用

将正常+黄芪（黄芪注射液 0.2g）组、正常+黄芪甲苷(0.4mg)组小鼠与正常对照组小鼠进行比较，观察其一般情况、有无死亡，一周后杀死，取心脏，4%多聚甲醛固定，石蜡包埋切片，片厚 4μm，行 HE 染色观察心肌有无病变。

（四）建立小鼠柯萨奇 $B_3$ 病毒性心肌炎模型

1、病毒: 柯萨奇 $B_3$ 病毒，Nancy 株，本实验室提供，Vero 细胞增殖后 ，测病毒滴度为 $10^{10}TCID_{50}$ 。

2、实验动物；清洁级雄性 4 周龄 BALB/C 小鼠，由上海医科大学实验动物中心提供。

3、动物模型制备：实验组小鼠腹腔接种 0.1ml 含 $10^5$ $TCID_{50}$ 柯萨奇 B3 病毒的 Eagle'液；对照组小鼠，每只腹腔接种 0.1ml 不含病毒的 Eagle'液。观察小鼠进食、毛色、活动及有无死亡，接种后第 7 天，处死存活小鼠，取其心脏，行常规病理检查、原位杂交和免疫组化检查。

（五）观察药物疗效

比较治疗组与非治疗组小鼠的死亡率、患病率，心肌病理变化，病理结果定量分析，比较各组心肌炎变面积百分比。

（六）免疫组化法检测小鼠心肌中颗粒酶 B 的表达

结果：

（一）药物毒性作用显示正常+黄芪组、正常+黄芪甲苷 0.4mg 组小鼠与正常对照组相似，一般情况均良好，小鼠无死亡，心肌病理检查未见病变。

（二）小鼠心肌炎病变情况，病毒感染组小鼠较正常组小鼠一般情况差，少食少动，部分小鼠死亡。心脏病理检查示不同程度的心肌炎症改变。

表 1 为小鼠心肌病变面积的比较

| 分组 | 小鼠序号 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 病毒对照 | 1 | 0.5 | 40 | 1 | 15 | 70 | 30 | 3 | 60 | |
| 病毒+黄芪 | 3 | 5 | 0 | 30 | 10 | 1 | 50 | 8 | | |
| 病毒+黄芪甲苷 0.1mg | 25 | 1 | 0 | 1 | 55 | 3 | 7 | | | |
| 病毒+黄芪甲苷 0.2mg | 0 | 10 | 6 | 10 | 3 | 20 | 1 | 1.5 | 1 | |
| 病毒+黄芪甲苷 0.4mg | 4 | 0 | 15 | 1 | 1 | 4 | 8 | 0 | 0 | 6 |

结果显示未治疗组小鼠心肌病变较重者构成比较大，心肌病变较轻者构成比较小。黄芪甲苷 0.2 mg 、黄芪甲苷 0.4 mg 治疗组心肌病变较未治疗组轻，$p < 0.05$。

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表 2：小鼠心肌病变面积频数分布表

| 分组 | 病变面积 (%) 0-25 | 25-50 | 50-75 | 75-100 | 合计 |
|---|---|---|---|---|---|
| 病毒对照 | 5 | 2 | 2 | 0 | 9 |
| 病毒+黄芪 | 6 | 1 | 1 | 0 | 8 |
| 病毒+黄芪甲苷 0.1mg | 5 | 1 | 1 | 0 | 7 |
| 病毒+黄芪甲苷 0.2mg | 9 | 0 | 0 | 0 | 9 |
| 病毒+黄芪甲苷 0.4mg | 10 | 0 | 0 | 0 | 10 |

表 2 可见治疗组小鼠心肌病变面积有下降的趋势，特别是黄芪甲苷 0.2 mg 治疗组和黄芪甲苷 0.4 mg 治疗组，存活小鼠的心肌病变面积均〈25% 。将心肌病变面积 25%为分界线，分别计算各组心肌病变轻重构成比。黄芪甲苷 0.2 mg 治疗组心肌病变轻重构成比为 9/0，与病毒对照组 5/4 相比，有显著性差异，p =0.04 。黄芪甲苷 0.4 mg 治疗组心肌病变轻重构成比为 10/0 ，与病毒对照组 5/4 相比 ，有显著性差异，p = 0.03 ，其余各组心肌病变面积轻重构成比与病毒对照组相比无显著性差异。

（三）免疫组化检查显示颗粒酶 B 的表达水平与病变面积呈正相关，治疗组（黄芪甲苷 0.2 mg、黄芪甲苷 0.4 mg 组）心肌病变面积小，颗粒酶 B 的表达水平也低，无心肌炎病理表现的小鼠心肌中未检出颗粒酶 B 的表达。

二、黄芪甲苷对小鼠病毒性心肌炎的心功能影响

(1) 建立小鼠病毒性心肌炎模型，感染病毒后取 VMC 小鼠行超声心动图检查，指标有左室缩短分数 FS，主动脉血流峰值流速 Vp，主动脉流速积分 Vi，存活小鼠超声检查后处死行心脏病理检查，观察各心功能指标的动态变化及病理变化。

（2）小鼠 VMC 模型，分为黄芪甲苷组、黄芪组、空白对照组和正常对照组。注射病毒后第 7 天，存活的小鼠行超声心动图检查，心脏病理评分。同时 ELISA 法测定血清肌钙蛋白含量；所有小鼠以病理积分分组，<1.5 为轻症组，≥1.5 为重症组，心脏中段石蜡切片

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进行原位杂交检测病毒 RNA，计算光镜下阳性信号占总面积的百分数。

结　果:

(1)VMC 组死亡小鼠，心功能指标较正常对照组低

(2) VMC 组 FS、Vp、Vi 均较对照-正常组降低，提示病毒感染组心功能减退；不用药的病毒感染组心功能减退明显（$p<0.05$）。

(3)病理评分：对照-VMC 组坏死面积较大；黄芪甲苷-VMC 组坏死较小，炎症浸润为主；黄芪-VMC 组病理改变与黄芪甲苷-VMC 组类似；不用药组较用药的两组者病变重（$p<0.05$）。对照-正常组和黄芪甲苷-正常组无病变，病理积分为 0；与前三组相比有显著性差异（$p<0.05$）。

结果证实黄芪甲苷能减轻 VMC 小鼠心肌炎症坏死程度，改善心功能。

三、黄芪甲苷对离体病毒性心肌炎细胞的保护作用

1. 选择病毒：选择柯萨奇 B 组病毒 B3 型作为感染原。在乳鼠心肌细胞上用 Reed 法测其 50%组织感染率(TCID50)。

2. 制备培养心肌细胞：取 Sprague-Dawley 大鼠(由上海医科大学实验动物部提供)，心室肌用 0.1％胰蛋白酶分次消化细胞，所得的细胞悬液纯化培养，生长液用含 20％小牛血清的 Eagle's MEM 液。

3. 观察病毒对培养搏动心肌细胞的感染及形态学变化：将已呈规律搏动的心肌细胞，加入 100 TCID50 的柯萨奇 B3 病毒适量，置 37℃吸附 1 小时后，观察其变化。结果显示接种病毒 2 天后，心肌细胞搏动百分比明显下降。CPE 很快自+～4+，细胞明显变园、成堆、有折光并团缩。CPE 表示法：+表示＜25%CPE，1+表示 25%CPE，2+表示 50%CPE，3+表示 75&CPE，4+表示近乎 100% CPE。

4.药物处理：将制备的培养心肌细胞，分组为：正常对照、病毒对照、病毒+黄芪甲苷共三组，正常组与病毒感染组只加上述生长液，在黄芪处理组加入含 0.1mg/ml 浓度的生长液；置 37℃孵育，在倒置相差显微镜下观察记录。

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结果显示出黄芪甲苷对CVB3感染的心肌细胞不论是细胞搏动%、细胞的形态均具有良好的保护作用。

附图说明:

图1为黄芪甲苷对离体病毒性心肌炎细胞的保护作用

# 说　明　书　附　图

图 1

病毒+黄芪甲苷处理

病毒+黄芪注射液对照

病毒对照

正常黄芪对照

正常细胞